

**Current Biology Volume 20**

**Supplemental Information**

**Stereotypical Cell Division Orientation**

**Controls Neural Rod Midline Formation**

**in Zebrafish**

**Elena Quesada-Hernández, Luca Caneparo, Sylvia Schneider, Sylke Winkler, Michael Liebling,  
Scott E. Fraser, and Carl-Philipp Heisenberg**



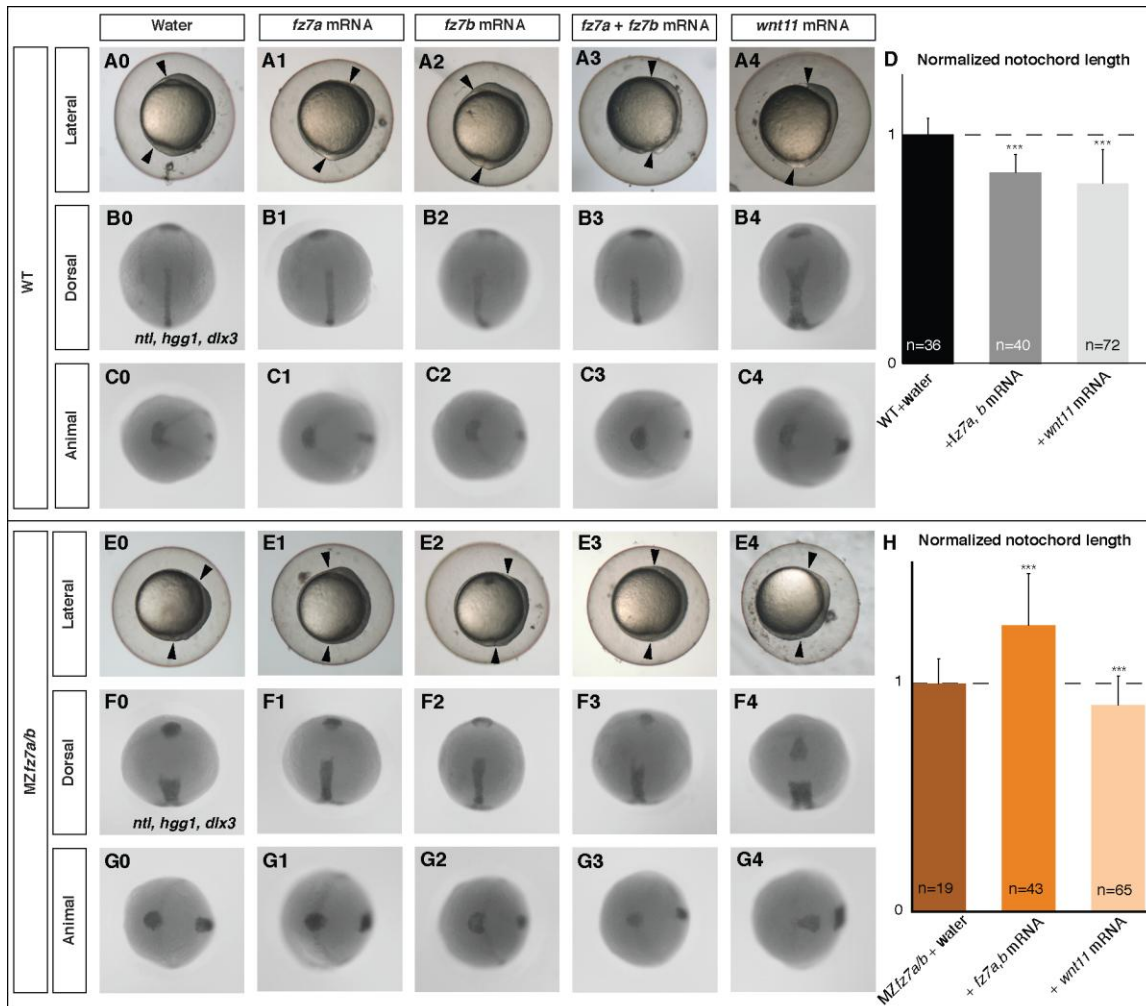
leading to truncated versions of the resulting peptides. The green box marks the position of the *Drosophila* mutant *fz<sup>15</sup>* that was classified as an amorphic allele [1].

(B) Sequencing of *fzd7a<sup>e3</sup>* allele revealed a T to A transversion in the sequence of the *fzd7a* locus leading in a nonsense mutation at position 1113 of the *fzd7a* cDNA. The resulting Fzd7a peptide is truncated at position 260.

(C) The *fzd7b<sup>hu3495</sup>* allele is caused by a C to T transition in the *fzd7b* locus that results in a nonsense mutation at position 1783 of the cDNA. The Fzd7b peptide is truncated at position 477.

(D) Truncated peptides resulting from the identified nonsense mutations of *fzd7a* and *fzd7b*.

(E) Phylogenetic tree of zebrafish and *Xenopus* Frizzled peptides shows that *fzd7* homologs of zebrafish and *Xenopus* cluster together (grey box) in one group. This tree was generated with JTT model of sequence evolution and gamma distributed rates across sites (MrBayes 3.1.2).



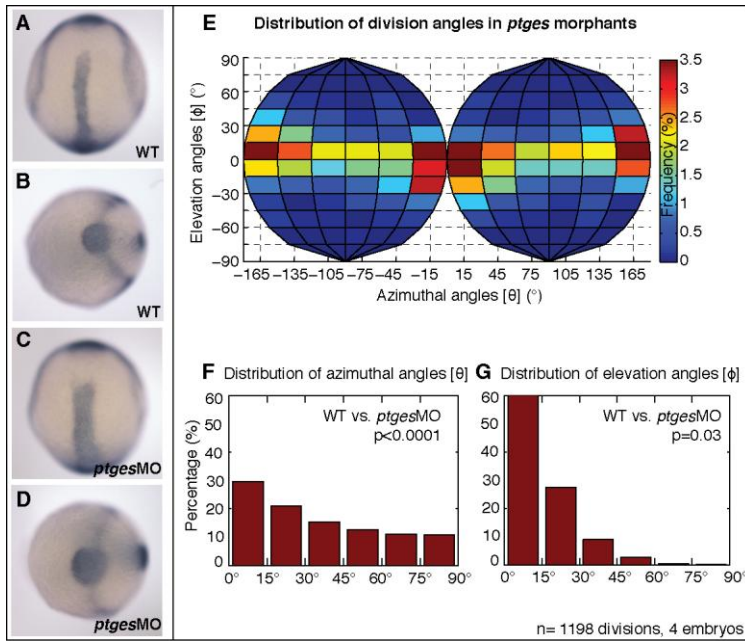
**Figure S2, related to Figure 1.** Fz7 functions downstream of Wnt11 in regulating convergence and extension movements and body axis elongation during gastrulation.

Columns **0 - 4** show wt (A-C) and MZfz7a/b (E-G) embryos injected with water (column 0), 100pg *fz7a* mRNA (column 1), 100pg *fz7b* mRNA (column 2), a mixture of 50 pg *fz7a* and 50 pg *fz7b* mRNA (column 3), and 100 pg *wnt11* mRNA (column 4).

Rows **B, C, F** and **G** show expression of *ntl*, *hgg1* and *dlx3* in wt (B,C) and MZfz7a/b (F,G) embryos. Dorsal (rows B,F) and animal views (rows C,G).

**(D, H)** Quantification of body axis length of wt (D) and MZfz7a/b (H) embryos injected with water, a mixture of 50 pg of *fz7a* and *fz7b* mRNAs, and 100pg of *wnt11* mRNA.

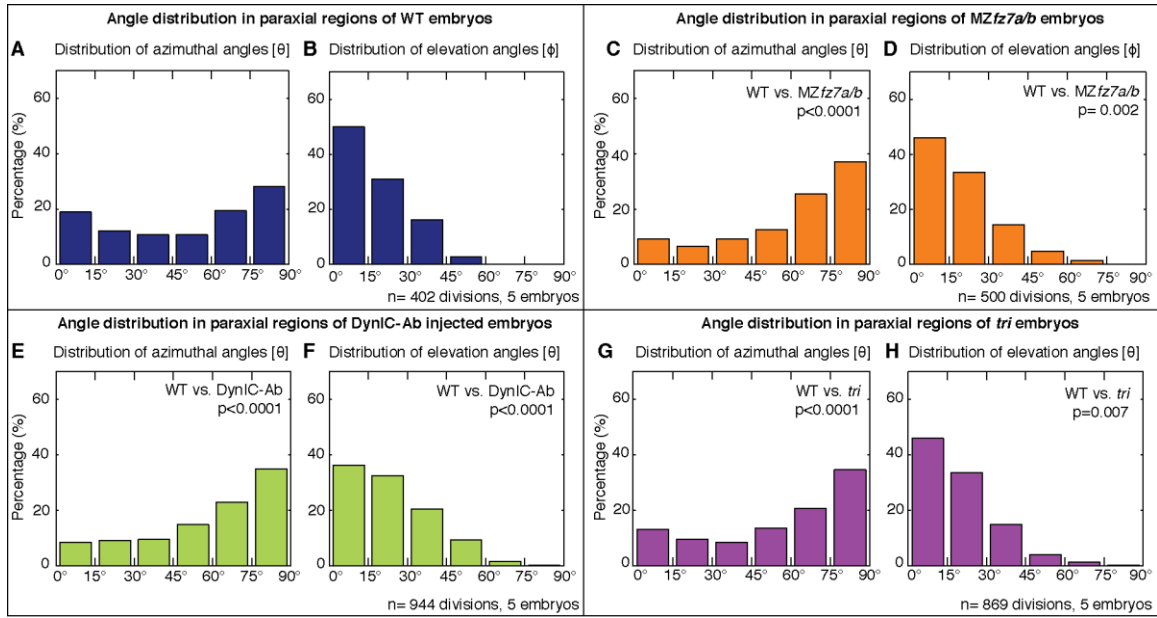
Error bars are standard deviation. \*\*\*=  $p < 0.001$



**Figure S3, related to Figure 2.** Stereotypical germ layer progenitor cell division orientation during gastrulation is mildly affected in embryos with defective convergence and extension movements, but intact Wnt/PCP signaling.

**(A-D)** Expression of *ntl*, *hgg1*, and *dlx3* marking the notochord, prechordal plate and anterior neural plate, respectively, in a wt (A,B) and *ptges* morphant embryo (C,D) at bud stage (10 hpf). Dorsal (A,C) and animal (B,D) views.

**(E-G)** Frequency distribution of cell division orientation angle pairs ( $\theta, \phi$ ) in *ptges* morphant embryos during gastrulation (6 - 10 hpf). Chi-squared test on the combined angles: WT vs. *ptges*MO ( $p = 0.0002$ ). Distribution of azimuthal ( $\theta$ ) (F) and elevation angles ( $\phi$ ) (G).



**Figure S4 related to Figure 3. Stereotypical neural progenitor cell division**

orientation is reduced in paraxial compared to axial regions of the forming neural rod in wild type, *MZfz7a/b* DynIC-Ab injected and *tri* embryos.

(A-H) Frequency distribution of cell division orientation angle pairs ( $\theta, \phi$ ) in paraxial regions of the forming neural rod in wt (A,B), *MZfz7a/b* (C,D), DynIC-Ab injected (E,F) and *tri* (G,H) embryos during neurulation (13 - 16 hpf).

Chi-squared test on the combined angles: wt axial vs. wt paraxial ( $p < 0.0001$ ), wt vs. *MZfz7a/b* ( $p = 0.015$ ), wt vs. DynIC-Ab ( $p = 0.018$ ), wt vs. *tri* ( $p = 0.095$ ).

## **Supplemental Experimental Procedures**

### *Embryo staging and maintenance*

Fish maintenance and embryo staging were carried out as described [2]. Details can be provided in request.

### *Generation of *fzd7a* and *fzd7b* mutant zebrafish*

Zebrafish libraries containing efficiently mutagenized individuals were successfully screened for point mutations in the coding sequence of *fzd7a* and *fzd7b* by re-sequencing of PCR fragments [3]. Two different zebrafish libraries that were generated in the context of the ZF model KO project were available for screening (libraries Dresden2: 4608 individuals and hubrecht34-48: 8832 individuals; ZF-models KO project: <http://www.zf-models.org/workpackages/wp4.html>). Point mutations in these libraries were chemically induced by the mutagen N-nitrosoethylurea (ENU) following standard protocols [4, 5]. To receive the genomic sequence of the *fzd7a* gene the respective cDNA sequence (NM\_131139.1) was blasted against the zebrafish ensembl database ([http://www.ensembl.org/Danio\\_rerio/index.html](http://www.ensembl.org/Danio_rerio/index.html)). The *fzd7a* cDNA is found on chromosome 9 (12906910-12909637). Based on this information, the organization of the *fzd7a* locus was manually annotated. *Fzd7b* (NM\_170763.1) is located on chromosome 6 (10693900-10697774) and the predicted annotation covers the whole cDNA. Genomic regions of interest were amplified by a nested PCR approach. Primer design was performed using a customized version of Primer3 that is integrated into the LIMSTILL interface (<http://limstill.niob.knaw.nl/index.html>). Oligonucleotides were designed against *fzd7a* (outer primer: CAAACCCGACGCCCTAC and ATGAGGTACCGATGAAGAGG, inner primer: TGTAACGACGGCCAGTATTACAGCCAAACCTGGTG and AGGAAACAGCTATGACCATTAGATGCCAACGTAGCAAAC) and against *fzd7b* (outer primer: TCTACAATGTTGACGCACTG and ATCAGCAAATGTGTGACCTG, inner primer:

TGTAACGACGGCCAGT CTTCTGGCTGGATTTGTTTC and AGGAAACAGCTATGACCAT TCAGTTCACACCGTTGTTTC). PCR conditions were 94°C for 2 min, followed by 35 cycles 94°C for 20 sec, 57°C for 30 sec, and 72°C for 1 min, and 72°C for 3 min. The second PCR product was processed as described in [6] and sequenced with the M13Forward primer (TGTAACGACGGCCAGT) that is linked to the inner forward PCR primer. All liquid handling steps were performed under high-throughput conditions using a TECAN Genesis workstation in 384-well format. Sequencing reactions as well as sequencing runs were performed at the sequencing facility of the Sanger Institute, Cambridge, England on AB3730XL DNA analyzers. Sequencing reads were automatically screened for point mutations with the Polyphred package version4 [7]. Primary hits were re-sequenced in an independent approach and verified point mutations were translated to see a potential effect, promising mutants were propagated further and bred to homozygosity.

#### *Fish lines*

Following zebrafish lines were used in this study: Wild type TL, H2A.F/Z:GFP ([8], kindly provided by M. Brand), *emi1*, *tri* [9] and MZ*fz7a/b*. Embryos were raised at 31°C, manipulated in E3 embryo water and staged as previously described [10].

#### *Cell division orientation analysis*

Dividing cells in H2A.F/Z:GFP transgenic or Histone H1-Alexa488 injected embryos were identified by the specific rearrangement of DNA during mitosis. Daughter cells were manually marked using the *Spots* object of Imaris (Bitplane, Inc.). Azimuthal and elevation angles ( $\theta, \varphi$ ) of the direction in which division occurs, as defined by the vector joining the center of marked sister cell pairs, were determined using a custom Matlab extension to Imaris that implements 3D inner products with the unit vectors along the main axes of the embryo (see Fig. 10). The angle pairs were binned in 2D histograms (12 bins along the azimuthal direction and 12 bins along the elevation direction) or in 1D aggregates (24 bins along the azimuthal direction



and 12 bins along the elevation direction). To compare distributions from two histograms, a chi-square test was applied to the binned histograms.

#### *Antibody, morpholino oligonucleotide, mRNA and dye injections*

To mark cell divisions, *MZfz7a/b* one-cell-stage embryos were injected with 0.5ng of Histone H1-Alexa488 conjugate (H13188, Invitrogen). To randomize cell division orientation, a mouse anti-Dynein Intermediate Chain 70.1 monoclonal antibody (D5167, Sigma) was injected at a 1:4 dilution into one-cell stage wild type embryos. Morpholino oligonucleotide (MO) against *ptges*, was ordered from Gene Tools and 4ng per embryo were injected at the one-cell-stage. mRNA was synthesized as previously described [11].

#### *Cell division inhibition*

To block cell division during gastrulation, embryos were incubated in E3 medium containing 100μM aphidicolin (Sigma) in DMSO and 20 mM hydroxyurea (Sigma) [12] from sphere to bud stages (4 – 10 hpf). To block cell division during neurulation, embryos were incubated in the same solution from 90% epiboly to the 16-somite-stage (9 - 17hpf).

#### *Image acquisition*

Embryos at shield (6 hpf) and 5-somite stage (12 hpf) were manually dechorionated and mounted in 1% low-melting point agarose prior to imaging. Time-lapse, multiple focal plane (4D) microscopy was performed at 28.5°C on a Bio-Rad Radiance 2000 multi-photon microscope. Fixed embryos at 16-somite-stage and stained for PHH3 and/or F-actin were mounted in 1% low melting point agarose and imaged at room temperature on a Zeiss LSM 510 confocal microscope using a Zeiss EC Plan-Neofluar 40x 1.3 oil immersion lens. Movies and Z-stacks were processed using Volocity (Improvision), Imaris (Bitplane), ImageJ, and Fiji software.

#### *In situ hybridization*

RNA *in situ* hybridization was performed as described [11]. Details can be provided upon request.

#### *Rhodamine-Phalloidin and Phospho-Histone H3 stainings*

Embryos at the 16-somite-stage were fixed with 4% paraformaldehyde (PFA) and manually dechorionated. Rabbit anti-Phospho Histone H3 (PHH3) antibody (1:300, Upstate Biotech) was used with goat anti-rabbit Alexa Fluor 488 (1:200, Molecular Probes). F-actin was detected using Rhodamine-Phalloidin (1:200, Invitrogen).

#### **Supplemental References**

1. Jones, K.H., Liu, J., and Adler, P.N. (1996). Molecular analysis of EMS-induced frizzled mutations in *Drosophila melanogaster*. *Genetics* *142*, 205-215.
2. Westerfield, M. (2000). *The Zebrafish Book*. University of Oregon Press *Edition 4*.
3. Wienholds, E., Schulte-Merker, S., Walderich, B., and Plasterk, R.H. (2002). Target-selected inactivation of the zebrafish *rag1* gene. *Science* *297*, 99-102.
4. Mullins, M.C., and Nusslein-Volhard, C. (1993). Mutational approaches to studying embryonic pattern formation in the zebrafish. *Curr Opin Genet Dev* *3*, 648-654.
5. Solnica-Krezel, L., Schier, A.F., and Driever, W. (1994). Efficient recovery of ENU-induced mutations from the zebrafish germline. *Genetics* *136*, 1401-1420.
6. Winkler, S., Schwabedissen, A., Backasch, D., Bokel, C., Seidel, C., Bonisch, S., Furthauer, M., Kuhrs, A., Cobreros, L., Brand, M., et al. (2005). Target-selected mutant screen by TILLING in *Drosophila*. *Genome Res* *15*, 718-723.
7. Nickerson, D.A., Tobe, V.O., and Taylor, S.L. (1997). PolyPhred: automating the detection and genotyping of single nucleotide substitutions using fluorescence-based resequencing. *Nucleic Acids Res* *25*, 2745-2751.
8. Pauls, S., Geldmacher-Voss, B., and Campos-Ortega, J.A. (2001). A zebrafish histone variant H2A.F/Z and a transgenic H2A.F/Z:GFP fusion protein for *in vivo* studies of embryonic development. *Dev Genes Evol* *211*, 603-610.
9. Hammerschmidt, M., Pelegri, F., Mullins, M.C., Kane, D.A., Brand, M., van Eeden, F.J., Furutani-Seiki, M., Granato, M., Haffter, P., Heisenberg, C.P., et al. (1996). Mutations affecting morphogenesis during gastrulation and tail formation in the zebrafish, *Danio rerio*. *Development* *123*, 143-151.
10. Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., and Schilling, T.F. (1995). Stages of embryonic development of the zebrafish. *Dev Dyn* *203*, 253-310.
11. Montero, J.A., Carvalho, L., Wilsch-Brauninger, M., Kilian, B., Mustafa, C., and Heisenberg, C.P. (2005). Shield formation at the onset of zebrafish gastrulation. *Development* *132*, 1187-1198.

12. Lyons, D.A., Pogoda, H.M., Voas, M.G., Woods, I.G., Diamond, B., Nix, R., Arana, N., Jacobs, J., and Talbot, W.S. (2005). *erbb3* and *erbb2* are essential for schwann cell migration and myelination in zebrafish. *Curr Biol* 15, 513-524.